Chapter IV

A histology-based fish health assessment of *Oreochromis mossambicus* on exposure of herbicide-Pyrazonsulfuron Ethyl

Introduction

Agrochemical products use in agriculture is a very common worldwide practice that causes contamination of water bodies. It has been reported that the level of toxic compounds (Heavy metals, organic pollutants and pesticides) has increased alarmingly (Pereira *et al*., 2013 and Jorundsdottir *et al*., 2014). These material travels from field to ground water or surface water by water by the process of leaching or run-off. The persistence of some pesticide becomes detrimental (Tiryaki and Temur, 2010). One of the widely used agrochemical products is herbicide PE commercially known as Saathi. Herbicides are actively used in terrestrial and aquatic ecosystems to control unwanted weeds, and their use has generated serious concerns about the potential adverse effects of these chemicals on the non-target organisms in aquatic ecosystem. The exposure of nontarget aquatic organisms to PE formulations by pollution of rivers and marine environments through runoff is a concern because the compound is highly water-soluble and extensively used (Singh *et al*., 2012). Fish species are most sensitive to aquatic pollutants during their early life stages (Yancheva *et al.,* 2015). The indiscriminate use of herbicides, careless handling, accidental spillage or discharge of untreated effluents into natural waterways have harmful effects on fish population and other forms of aquatic life and may contribute long term effects in the environment (Ramah, 2011). The herbicides affect not only the physiology and survival of aquatic organisms including fish but also interact with their

genetic make-up leading to mutations and/or carcinogenesis (Akinsorotan *et al.*,2013, Cressey, 2015 and WHO, 2015).

Health of aquatic organisms cannot be measured directly. Instead, only indicators of health can be measured and in turn used to assess the ‘‘health’’ status. Histology and histopathology is used as biomonitoring tools or indicators of health in toxicity studies as they provide early warning signs of disease (Marchand *et al.,* 2009). Histopathological alterations are biomarkers of effect of exposure to environmental stressors, revealing prior alterations in physiological and/or biochemical function (Barnhoorn *et al.,* 2010). Fish is a suitable indicator for monitoring environmental pollution because they concentrate pollutants in their tissues directly from water and also through their diet, thus enabling the assessment of transfer of pollutants through the trophic web (Fisk *et al.,* 2001, Boon *et al.,* 2002). Due to being exposed to pollutants, major structural damages may occur in their target organs, histological structure may change and physiological stress may occur. This stress causes some changes in the metabolic functions. The changes in the functions are initiated with the changes in the tissue and cellular level. Although Quantitative data show reactions of the organisms to the pollutant, qualitative data are used in most cases to study the pathologies the environmental pollutants cause (Nibamureke *et al.,* 2016).

Histopathological study has been widely used for toxicity testing for the effects of xenobiotic compounds at the suborganismal or organismal level, as well as evaluation of overall health of the entire population in the ecosystem. Histopathological changes of organs such as the gills, kidneys and liver, have been described as biomarkers in the evaluation of the health of fish exposed to pollutants (Gerber *et al.,* 2016). They are responsible for vital functions and can alter

haematological parameters because of changes in their activity in response to various stress factors. Review on available literature on fish and environmental pollutants indicate that the sublethal doses of most of the pesticides cause varying extent of histopathological injuries to different organs in fishes; the amount of damages are usually dependent on dose, duration of exposure and type of pesticides (Desai and Parikh 2012., Sadekarpawar *et al.*, 2015 Bhagade, 2012; Agamy, 2012;). Histological and ultrastructural changes in the cells can thus be used as good biomarkers of pollutant stress. Moreover, histopathology makes it possible to detect both acute and chronic changes in the tissue of individual organisms (Stentiford *et al.,* 2003). The utility of histological lesions as sensitive and reliable indicators of fish health has been reported in previous studies (Ramírez-Duarte *et al.,* 2008, Desai and Parikh, 2016; Sadekarpawar and Parikh, 2015). Numerous studies of glyphosate-based herbicides (Roundup) have demonstrated toxic effects on fish (the Nile tilapia *Oreochromis niloticus* and *Jenynsia multidentata*), and reported that Roundup can cause histological changes in the liver, gills, and kidneys after acute and chronic exposure to sublethal concentrations (Ayoola, 2008; Langiano Vdo and Martinez, 2008; Hued *et al.*, 2012). However, no previous study has investigated the aquatic toxicity of PE with particular reference to histopathological analyses. The advantage of using histopathological symptoms in specific target organs like the gills, liver, and kidneys in environmental monitoring is that they are most effective to study the vital functions, such as respiration, accumulation and biotransformation, and excretion of xenobiotics in fish (Camargo and Martinez, 2007 ; Parikh *et al.*,2014 and Samanta *et al.*,2016). Histological changes appear as prime responses to sublethal stressors, and have long been recognized to be reliable biomarkers of stress in fish for several reasons (van der Oost *et al.*,2003), which ultimately serve as warning signs of damage to animal health (Thornton *et al.,* 2013, Naeemi *et al.*,2013 and Ghobadian *et al.,* 2017).

A need based demand of quantifying the histological alterations were first time reported by Bernet *et al.,* (1999) where he developed a standardised tool for the assessment of histological findings which was applied to different organs during the assessment for the effects of sub-lethal and chronic effects of water pollution on fish. The methodology was based on the extent (score value) and pathological importance (importance factor) of the change observed. The sum of the multiplied score values and importance factors of all changes result in different indices which then statistically analysed.

Semiquantitative approach has been reported to be used byZimmrrli *et al.*, 2007 and Van Dyk *et al.,* 2009a and Ackermen, 2008, where they have tried to compare the fish population of 2 differant fresh water streams to check the health status of fish. Further, A study on the testicular histology where reproductive health of C. gariepinus and *O. mossambicus* from a DDT sprayed area has also been well explored by Marchand *et al.*, 2010. Moreover, Histological alterations in the gonads and gills of *C. gariepinus* from an urban nature reserve in South Africa by Pieterse *et al.,* 2010b has also been analysed. The scoring system/semi-quantitative histological studies thus allow us to easily summarise the severity of alterations within a specific organ which can be compared with the control.

Keeping this in mind the aim of the present work was first to find out the histological alterations, semiquantify it and to determine the health of the O. mossambicus by comparing it with the control fish with different dose and at two different time period so at to understand the impact of PE on kidney, Liver and Gills .

Materials and Methods:

Experimental design:

Live and healthy male and female adult *O.mossambicus* was procured from the pure brooders of length 12±3cm and weight 25±3g. Fishes (5 males and 5 females) were kept in a clean glass aquarium for an acclimation period of 12-15 days in de-chlorinated water at 27 ± 4ºC, pH 7.4 ± 0.05, dissolved oxygen 8 ± 0.3 mg/Land total hardness 188 mg/L $CaCO_3$ with a 12:12 light: dark photoperiod. They were fed with the commercial available healthy food during the period of study. If in any batch, mortality exceeds 5% during acclimatization, that entire batch of fish was discarded. Animal maintenance and experimental procedures were in accordance with the guideline of A.P.H.A., A.W.W.A. and W.P.C.F. (1998).

Sub-lethal exposure:

On basis of 96 hr LC50 value sub-acute study dose 1/20th LC50 (Low dose), 1/10th LC50 (Medium dose), 1/5th LC50 (High dose) were chosen for Health assessment protocol studies. A control group was maintained. The experiment was performed semi statically with a group of 10 fish in triplicate, one control and one test aquaria. All the groups were kept under continuous observation during the experimental period. Commercially food pallets were given to fish once in day during the experiment adlibitum. Test chemical and test media were changed every 24 hours to maintain the toxicant strength and the level of dissolved oxygen as well as to minimize the level of ammonia during experiment.

- Group 1 served as control without any treatment of PE.
- Group 2 were treated with low dose of PE (1/20th LC50- Low Dose)

- Group 3 were treated with medium dose of PE (1/10th LC50 - Medium Dose)
- Group 4 were treated with high dose of PE (1/5thLC50 - High Dose)

At the end of the experiment the fish were carefully netted to minimize stress. They were slowly released in the tough containing 1% clove oil to make it immobile, and then after each fish was held and wrapped with a clean, dry towel and the posterior half of its body were blotted with a clean coarse filter paper. The caudal peduncle of the fish was severed with a single stroke from a heavy, sharp scissor. Then, the liver, kidney and gill organs for histological observations was carefully removed and weighed.

Histological observation:

After measuring weight fresh tissues were fixed in 4% paraformaldehyde for 24 hrs, dehydrated, embedded in paraffin wax and sectioned at 10-12µm then stained with heamatoxylin and eosin and examined microscopically and photographed using digital camera.

Semi-quantitative histology assessment:

A standard semi quantitative histology assessment was perform according to the modified protocol of van dyk *et al.,*2009 which were observe in gill, liver and kidney histology slides.

There are four reaction patterns describe by pieterse *et al.,* 2010, according to which each organ were assessed. The reaction patterns are

Reaction pattern 1: (RP 1): Circulatory disturbances (CD)

These include haemorrhage, hyperaemia, aneurysm and intracellular edema.

Reaction pattern 2: (RP 2): Regressive changes (RC)

The reaction patterns which are involved in architectural and structural alteration, plasma alteration, nuclear alteration, atrophy and necrosis.

Reaction pattern 3: (RP 3): Progressive changes (PC)

Characteristic lesion is hypertrophy or hyperplasia.

Reaction pattern 4: (RP 4): Inflammation (inf)

It includes exudates activation of reticulo-endothelial systems, infiltration.

Importance Factor (W):

The 3 important factors as different as follow:

1. Minimal pathological importance- the lesions is easily reversible as exposure to irritants ends.

2. Moderant pathological importance- the lesions is reversible in some cases if the stressor is neutralized.

3. Marked pathological importance- the lesions is irreversible leading to organ death.

Score Value (a):

Each alteration has been assign a score value from 0 to 6 by performing the assessment, depending on the degree and extends of alteration. (0) unchanged, (2) mild occurrence, (4) moderate occurrence that was alteration present in half of the tissue and (6) severe alteration that is present all over the tissue.

Using importance factors and score values, four different indices calculated. If the lesions within one organ only were studied, the two following indices were applicable:

- **Organ Index (Iorg)**

This index represents the degree of damage to an organ. This calculation allows a comparison between the degree of damage of the same organ in different individuals. It was calculated as follows:

Iorg = ΣrpΣalt (aorg rp alt x w org rp alt)

Where: org = organ (constant);

rp = reaction pattern;

alt = alteration;

a = score value;

w =importance factor.

This index was the sum of the multiplied importance factors and scorevalues of all changes found within the assessed organ.

- **Reaction index of an organ (Iorg rp)**

The reaction index expresses the quality of lesions within an organ. Respective reactionindices of an organ (Iorg rp) from different individuals can be compared. The reaction index was calculated as follows:

- **Iorg rp = Σalt (aorg rp alt x w org rp alt)**

Where: org, rp = constant.

This index was calculated by the sum of the multiplied importance factors and score values of the alterations of the corresponding reaction pattern.

The organ index results were separated into four classes according to the protocol of Van Dyk *et al.*,(2009a) which were modified from Zimmerli *et al.*,(2007).

The classes are as follows:

Class 1 (index< 10) –tissue with slight histological alterations

Class 2 (index 10-25) - tissue with moderate histological alterations

Class 3 (index 26-35) – pronounced alterations of organ tissue

Class 4 (index >35) – severe alterations of organ tissue

- **Fish Index (Ifish)**

The fish index represents a measure of the overall health status of the fish based on the occurrence of histological alterations observed in all of the organs. As this index is calculated in the same way for each fish, a comparison between individuals is possible. The fish index was calculated as follows:

Ifish = ΣorgΣrpΣalt (a org rp alt x w org rp alt)

Statistical Analysis

A high index is a representative of a high degree of organ or tissue damage. Mann-Whitney test were used to identify the statistical difference for the two different time period. Further, the Bonferroni corrections was applied in order to adjust the significance level to an alpha level of 1% was used when analysing the results ($p<0.05$).

Result:

The reaction index (I org rp) gives an indication of the quality of the lesions in the specific organ, and the organ index (Iorg) gives an indication to the degree of damage. Table 4.1 and 4.2 summarizes the quantitative results obtained after the exposure of PE for 7 and 14 days at high, medium and low doses. Time and dose dependent alteration in the reaction pattern was obtained for Gills, Liver and Kidney.

Gills: As far as gills are concerned the circulatory disturbance, regressive changes were comparatively higher only after 14th day of exposure. The progressive changes were not that significant. The organ index (I org) of gills at low dose was 0 followed by 7 at the medium dose and 9 at high dose after 7th days of exposure, signifying that the gills tissues showed slight histological alterations. After 14th day the low dose of PE exposure gill organ index (I org) was 14 followed by 27 at medium dose and 38 at high dose, signifying that the gill tissue had moderate, pronounced to severe alterations.

Liver: The organ index (I org) of liver at low dose was 5 followed by 10 at medium dose and 13 at high dose after 7th days of PE exposure, signifying that the liver tissues showed slight to moderate histological alterations. After 14th day the low dose of PE exposure liver organ index (I org) was 21 which comes under moderate alteration suggesting that the lesions can be reversible if the fishes were allowed for recovery. At medium dose the liver organ index was 31 followed by 43 at high dose with pronounced and severed histological alterations respectively, suggesting that these alterations will be difficult to get back to normal even after recovery (Smith, 2012).

Kidney: The organ index (I org) of kidney at low dose was 5 followed by 8 at medium dose and 10 at high dose after 7th days of exposure, signifying that the kidney tissues showed slight to moderate histological alterations. After 14th day the low dose of PE exposure kidney organ index (I org) was 18 at low dose followed by 31 at medium dose and 43 at high dose, signifying that the kidney tissue had moderate, pronounced to severe alterations which are irreversible changes probably leading to partial or total loss of organ function.

As seen in Table 4.1 and 4.2, the organ with highest index, and thus the highest degree of damage, was the kidney. The Kidney of the highest dose on 14th day of exposure group was significantly higher than the kidney of the highest dose on 7th day of exposure ($p<0.01$). The liver and gills on the other hand was moderately significant ($p<0.5$).

Micrograph of kidney, liver and gills are displayed in Figs. 4.1 to 4.6.

The normal histological structure of the kidney of *O. mossambicus* consists of nephrons surrounded by haematopoietic tissue. The nephrons were made up of renal corpuscles (Fig.4.3) and renal tubules. The renal corpuscle which is made up of a glomerulus is surrounded by Bowman‘s capsule. The glomerulus was found to consist of a cluster of blood capillaries filled with red blood cells. A clear cut lumen (Bowman‘s space) was also observed (Fig). Proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) were also clearly distinguished.

All the three reaction pattern in the form of histopathological alterations were observed i.e. circulatory disturbances in the form of hyperaemia and dilation of glomeruli capillaries; regressive changes were observed where the structural alterations in DCT, PCT, glomerulus along with a well distinguished altered interstitial tissue. The progressive changes were well distinguished in tubules only. Thus signifying a prominent regressive alteration.

The normal histological structure of the liver of *O. mossambicus* consists of hepatocytes arranged in hepatic cords along with blood sinusoid (Fig. 4.1 and 4.2). Along with clear liver section, hepatopancreatic tissue (Fig 4.2) was also observed and was identified as dark purple stained with H and E pancreatic acini with a large nucleus and pink stained zymogen granules. The PE exposure resulted in a dose and time dependent alterations with distinguished reaction pattern. Where the circulatory disturbances were observed by hyperaemia; the regressive changes were evident by the overall changes in the hepatocytes, bile duct and an increase in MMCs; he progressive changes were hyperplasia and hypertrophy in the hepatocytes.

In all the control fish, as far as gills are concerned, the normal histology structure of gills consists of primary lamellae which are supported by various cell types such as lymphocytes, undifferentiated cells and well defined epithelium lining; the secondary lamellae consist of rodlet cells, chloride cells, pillar cells and blood vessels (Fig. 4.5). The remarkable alterations in the reaction pattern were observed on PE exposure. Of the three reaction pattern the most pronounced were the regressive changes in which the epithelium and the supporting tissues in which the curling of the secondary lamellae along with the disappearance of the supporting (Fig.4.5).

Table 4.1: Summary of the reaction index (Iorg rp), Organ index (I org), Total reaction index (I rp) and the total index (Tot-I) of the selected organ as calculated for the groups 1/20th LC_{50}, 1/10th LC_{50} and1/5th LC_{50} for the 7th days exposure of PE. (CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I-Inflammation)

	Gill			Liver			Kidney		
Reaction pattern	**1/20th LC_{50}**	**1/10th LC_{50}**	**1/5th LC_{50}**	**1/20th LC_{50}**	**1/10th LC_{50}**	**1/5th LC_{50}**	**1/20th LC_{50}**	**1/10th LC_{50}**	**1/5th LC_{50}**
Iorg rp									
CD	0	2	2	0	1	1	1	1	2
RC	0	3	5	5	9	10	3	5	6
PC	0	2	2	0	0	2	2	2	2
Iorg	**0**	**7**	**9**	**5**	**10**	**13**	**5**	**8**	**10**

Table4.2: Summary of the reaction index (Iorg rp), Organ index (I org), Total reaction index (I rp) and the total index (Tot-I) of the selected organ as calculated for the groups 1/20th LC_{50}, 1/10th LC_{50} and1/5th LC_{50} for the 14th days exposure of PE. (CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I-Inflammation)

	Gill			Liver			Kidney		
Reaction pattern	**1/20th LC_{50}**	**1/10thLC_{50}**	**1/5th LC_{50}**	**1/20th LC_{50}**	**1/10th LC_{50}**	**1/5th LC_{50}**	**1/20th LC_{50}**	**1/10th LC_{50}**	**1/5th LC_{50}**
Iorg rp									
CD	3	4	6	1	2	2	2	4	6
RC	9	20	24	16	24	32	14	25	32
PC	2	3	8	4	5	9	2	2	5
Iorg	**14**	**27**	**38**	**21**	**31**	**43**	**18**	**31**	**43**

Table 4.3: Gill tissue Histological assessment sheet of *O. mossambicus* on exposure of PE 7th day

Gill Tissue (**LD, MD, HD**)- 7th day												
RP	Functional Unit	Alterations	Pathological condition	a	W	a x w	a	W	a x w	a	w	a x w
CD		Aneurysm/Hyperaemia/Haemorrhage	e.g. congestion	0	0	0	1	1	1	1	1	1
			e.g. telangiecstasia	0	1	0	1	1	1	1	1	1
		Intercellular oedema		4	2	0	0	0	0	0	1	0
			RP INDEX			**00**			**02**			**02**
RC	Epithelium	Structural alterations	e.g. sec lam branching	0	0	0	1	1	1	2	1	2
		Plasma alterations	Vacuolation	0	0	0	0	0	0	0	0	0
		Inter cellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	0	0	0	0	0	0
		Rupture of pillar cells		0	0	0	0	0	0	0	0	0
		Atrophy		0	0	0	1	1	1	1	1	1
		Necrosis		0	0	0	1	1	1	1	2	2
	Supporting tissue	Structural alterations	e.g. prim lam branching	0	0	0	0	1	0	0	1	0
		Plasma membrane alterations		0	0	0	0	1	0	0	1	0
		Intercellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	0	0	0	0	0	0
		Atrophy		0	0	0	0	0	0	0	0	0
		Necrosis		0	0	0	0	0	0	0	0	0
			RP INDEX			**00**			**03**			**05**
PC	Epithelium	Hypertrophy		0	0	0	1	1	1	1	1	1
		Hyperplasia		0	0	0	1	1	1	1	1	1
			RP INDEX			00			02			02

Table 4.4: Gill tissue Histological assessment sheet of *O. mossambicus* on exposure of PE 14th day

Gill Tissue (**LD, MD, HD**)- 14th day												
RP	Functional Unit	Alterations	Pathological condition	a	W	a x w	a	W	a x w	a	w	a x w
CD		Aneurysm/Hyperaemia/Haemorrhage	e.g. congestion	1	1	1	1	1	1	1	1	1
			e.g. telangiecstasia	1	1	1	2	1	2	2	2	4
		Intercellular oedema		1	1	1	1	1	1	1	1	1
			RP INDEX			**03**			**04**			**06**
RC	Epithelium	Structural alterations	e.g. sec lam branching	2	1	2	2	1	2	2	1	2
		Plasma alterations	Vacuolation	0	0	0	0	0	0	1	1	1
		Inter cellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	1	1	1	1	1	1
		Rupture of pillar cells		0	0	0	1	1	1	1	2	2
		Atrophy		1	2	2	2	2	4	2	2	4
		Necrosis		1	2	2	2	2	4	2	2	4
	Supporting tissue	Structural alterations	e.g. prim lam branching	1	1	1	2	2	4	2	2	4
		Plasma membrane alterations		0	1	0	0	1	0	1	1	1
		Intercellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	0	0	0	1	1	1
		Atrophy		1	1	1	1	2	2	1	2	2
		Necrosis		1	1	1	1	2	2	1	2	2
			RP INDEX			**09**			**20**			**24**
PC	Epithelium	Hypertrophy		1	1	1	1	2	2	2	2	4
		Hyperplasia		1	1	1	1	1	1	2	2	4
			RP INDEX			02			03			08

Table 4.5: Liver tissue Histological assessment sheet of O. mossambicus on exposure of PE 7th day

Liver(**LD, MD, HD**)- 7th day				LD			MD			HD		
RP	Functional Unit	Alterations		a	w	a x w	A	w	a x w	a	w	a x w
CD		Aneurysm/Hyperaemia/Haemorrhage	e.g. induce congestion	0	1	0	1	1	1	1	1	1
		Intercellular oedema		0	0	0	0	0	0	0	0	0
			RP INDEX			0			**1**			1
RC	Liver tissue	Structural alterations	e.g. cord disarray & cell structure	0	1	0	1	1	1	1	1	1
	Hepatocytes	Plasma membrane alterations	Granular degeneration / intra cellular deposits	0	0	0	0	0	0	0	0	0
			Fatty degeneration (e.g. fatty change)	0	0	0	0	0	0	0	0	0
			Glycogen vacuoles	0	0	0	0	0	0	0	0	0
			Vacuolation (content unknown)	0	0	0	0	0	0	0	0	0
		Inter cellular deposits		0	0	0	0	0	0	0	0	0
		Increase in MMC		0	0	0	0	0	0	0	0	0
		Nuclear alterations	Pleomorphism / chromatin clearing	0	0	0	0	0	0	0	0	0
			Pyknosis	0	0	0	0	0	0	0	0	0
		Atrophy		1	1	1	1	1	1	1	1	1
		Necrosis		1	1	1	1	1	1	1	1	1
	Interstitial tissue	Structural alterations		1	1	1	1	1	1	1	1	1
		Plasma alterations	Granular degeneration	0	0	0	0	0	0	0	0	0

			/ intra cellular deposits									
			Vacuolation	0	0	0	0	0	0	0	0	0
		Intercellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	0	0	0	0	0	0
		Atrophy		0	0	0	1	1	1	1	2	2
		Necrosis		0	0	0	1	1	1	1	1	1
	Bile ducts	Structural alterations		0	0	0	0	0	0	0	0	0
		Plasma alterations	Granular degeneration / intra cellular deposits	0	0	0	0	0	0	0	0	0
			Vacuolation	0	0	0	0	0	0	0	0	0
		Intercellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		1	1	1	1	1	1	1	1	1
		Atrophy		0	1	0	1	1	1	1	1	1
		Necrosis		1	1	1	1	1	1	1	1	1
			RP INDEX			5			**9**			10
PC	Liver tissue	Hypertrophy		0	1	0	0	1	0	1	1	1
		Hyperplasia		0	0	0	0	0	0	1	1	1
		Wall proliferation	e.g. blood vessels	0	1	0	0	1	0	0	1	0
	Interstitial tissue	Hypertrophy		0	0	0	0	0	0	0	0	0
		Hyperplasia	e.g. cirrhosis	0	0	0	0	0	0	0	0	0
	Bile ducts	Hypertrophy		0	0	0	0	0	0	0	0	0
		Hyperplasia		0	1	0	0	1	0	1	0	1
		Wall proliferation		0	0	0	0	0	0	0	0	0
			RP INDEX			0			**0**			2
I		Exudate		0	0	0	0	0	0	0	0	0
		Activation of RES		0	0	0	0	0	0	0	0	0
		Infiltration	e.g. leucocytes (MNL) - lymphocytes	0	0	0	0	0	0	0	0	0
			e.g. granulocytes	0	0	0	0	0	0	0	0	0
			RP INDEX			0			**0**			0

Table 4.6: Liver tissue Histological assessment sheet of *O. mossambicus* on exposure of PE 14th day

Liver(**LD, MD, HD**)- 14th day							MD			HD		
RP	Functional Unit	Alterations		a	w	a x w	A	w	a x w	A	w	a x w
CD		Aneurysm/Hyperaemia/Haemorrhage	e.g. induce congestion	1	1	1	1	2	2	1	2	2
		Intercellular oedema		0	0	0	0	0	0	0	0	0
			RP INDEX			**1**			**2**			**2**
RC	Liver tissue	Structural alterations	e.g. cord disarray & cell structure	1	2	2	2	2	4	2	2	4
	Hepatocytes	Plasma membrane alterations	Granular degeneration / intra cellular deposits	0	0	0	0	0	0	0	0	0
			Fatty degeneration (e.g. fatty change)	0	0	0	0	0	0	0	0	0
			Glycogen vacuoles	0	0	0	0	0	0	0	0	0
			Vacuolation (content unknown)	0	0	0	0	0	0	0	0	0
		Inter cellular deposits		0	0	0	0	0	0	0	0	0
		Increase in MMC		0	0	0	0	0	0	0	0	0
		Nuclear alterations	Pleomorphism / chromatin clearing	0	0	0	1	1	1	1	1	1
			Pyknosis	1	1	1	1	2	2	2	2	4
		Atrophy		1	1	1	1	1	1	1	1	1
		Necrosis		1	2	2	2	2	4	2	2	4
	Interstitial tissue	Structural alterations		1	1	1	1	1	1	1	1	1
		Plasma alterations	Granular degeneration / intra cellular deposits	0	0	0	0	0	0	0	0	0
			Vacuolation	0	0	0	0	0	0	0	0	0
		Intercellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	0	0	0	0	0	0
		Atrophy		1	2	2	1	2	2	1	2	2
		Necrosis		1	2	2	1	2	2	1	2	2
	Bile ducts	Structural alterations		1	1	1	1	2	2	2	2	4

		Plasma alterations	Granular degeneration / intra cellular deposits	0	0	0	0	0	0	0	0	0
			Vacuolation	0	0	0	0	0	0	0	0	0
		Intercellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations		1	2	2	1	2	2	2	2	4
		Atrophy		1	1	1	1	1	1	1	1	1
		Necrosis		1	1	1	1	2	2	2	2	4
			RP INDEX			**16**			**24**			**32**
PC	Liver tissue	Hypertrophy		1	1	1	1	1	1	1	1	1
		Hyperplasia		1	2	2	1	2	2	2	2	4
		Wall proliferation	e.g. blood vessels	0	1	0	0	1	0	0	1	0
	Interstitial tissue	Hypertrophy		0	0	0	0	0	0	0	0	0
		Hyperplasia	e.g. cirrhosis	1	1	1	1	1	1	1	1	1
	Bile ducts	Hypertrophy		0	0	0	1	1	1	2	1	2
		Hyperplasia		0	1	0	0	1	0	1	1	1
		Wall proliferation		0	0	0	0	0	0	0	0	0
			RP INDEX			**4**			**5**			**9**
I		Exudate		0	0	0	0	0	0	0	0	0
		Activation of RES		0	0	0	0	0	0	0	0	0
		Infiltration	e.g. leucocytes (MNL) - lymphocytes	0	0	0	0	0	0	0	0	0
			e.g. granulocytes	0	0	0	0	0	0	0	0	0
			RP INDEX			**0**			**0**			**0**

Table 4.7: Kidney tissue Histological assessment sheet of *O. mossambicus* on exposure of PE 7th day

Kidney Tissue- (**LD, MD, HD**)- 7th day							MD			HD		
RP	Functional Unit	Alterations		a	w	a x w	A	w	a x w	A	w	a x w
CD		Aneurysm/Hyperaemia/Haemorrhage	e.g. induce congestion	0	1	0	0	1	0	1	1	1
		Intercellular oedema		0	0	0	0	0	0	0	0	0
		Dilation of glomeruluscapillaries		1	1	1	1	1	1	1	1	1
			RP INDEX			**1**			**1**			**2**
RC	Tubule	Structural alterations		1	1	1	1	1	1	1	1	1
		Plasma alterations	Vacuolation	0	0	0	0	0	0	0	0	0
			Hyaline droplet degeneration	0	0	0	0	0	0	0	0	0
			Basophilic cytoplasm	0	0	0	0	0	0	0	0	0
			Granular degeneration	0	0	0	0	0	0	0	0	0
		Inter cellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations	Pleomorphism / chromatin clearing	0	0	0	0	0	0	0	0	0
			Pyknosis	0	0	0	1	1	1	1	1	1
		Atrophy		0	0	0	0	0	0	0	0	0
		Necrosis		0	0	0	0	0	0	0	0	0
	Interstitial tissue	Structural alterations		0	0	0	0	0	0	0	0	0
		Plasma membrane alterations	Granular degeneration / intra cellular deposits / vacuolation	0	0	0	0	0	0	0	0	0
		Intercellular deposits	MMC	0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	0	0	0	0	0	0
		Atrophy		0	0	0	0	0	0	0	0	0
		Necrosis		0	0	0	0	0	0	0	0	0
	Glomerulus	Structural alterations		1	1	1	1	1	1	1	2	2
		Plasma alterations	Granular	0	0	0	0	0	0	0	0	0

			degeneration / intra cellular deposits / vacuolation									
		Intercellular deposits	MMC	0	0	0	0	0	0	0	0	0
		Nuclear alterations		0	0	0	1	1	1	1	1	1
		Atrophy		0	0	0	0	0	0	0	0	0
		Necrosis		1	1	1	1	1	1	1	1	1
			RP INDEX			**3**			**5**			**6**
PC	Tubule	Hypertrophy	e.g. albuminous degeneration (reversible) (cloudy swelling)	0	0	0	0	0	0	0	0	0
		Hyperplasia		1	1	1	1	1	1	1	1	1
	Interstitial tissue	Hypertrophy		0	0	0	0	0	0	0	0	0
		Hyperplasia	e.g. cirrhosis	0	0	0	0	0	0	0	0	0
	Glomerulus	Hypertrophy		1	1	1	1	1	1	1	1	1
		Hyperplasia		0	0	0	0	0	0	0	0	0
		Thickening of BC membrane		0	0	0	0	0	0	0	0	0
			RP INDEX			**2**			**2**			**2**
I		Exudate		0	0	0	0	0	0	0	0	0
		Activation of RES		0	0	0	0	0	0	0	0	0
		Infiltration	e.g. leucocytes (MNL) - lymphocytes	0	0	0	0	0	0	0	0	0
			e.g. granulocytes	0	0	0	0	0	0	0	0	0
			RP INDEX			**0**			**0**			**0**

Table 4.8: Kidney tissue Histological assessment sheet of *O. mossambicus* on exposure of PE 14th day

Kidney Tissue- (**LD, MD, HD**)- 14th day							MD			HD		
RP	Functional Unit	Alterations		a	w	a x w	A	w	a x w	a	w	a x w
CD		Aneurysm/Hyperaemia/Haemorrhage	e.g. induce congestion	1	1	1	1	2	2	1	2	2
		Intercellular oedema		0	0	0	0	0	0	0	0	0
		Dilation of glomeruluscapillaries		1	1	1	1	2	2	2	2	4
			RP INDEX			**2**			**4**			**6**
RC	Tubule	Structural alterations		1	2	2	2	2	4	2	2	4
		Plasma alterations	Vacuolation	0	0	0	0	0	0	0	0	0
			Hyaline droplet degeneration	0	0	0	0	0	0	0	0	0
			Basophilic cytoplasm	1	1	1	1	1	1	1	1	1
			Granular degeneration	0	0	0	0	0	0	0	0	0
		Inter cellular deposits		0	0	0	0	0	0	0	0	0
		Nuclear alterations	Pleomorphism / chromatin clearing	1	1	1	1	2	2	1	2	2
			Pyknosis	1	1	1	1	2	2	1	2	2
		Atrophy		0	0	0	1	1	1	1	2	2
		Necrosis		1	1	1	1	2	2	1	2	2
	Interstitial tissue	Structural alterations		1	1	1	1	1	1	1	1	1
		Plasma membrane alterations	Granular degeneration / intra cellular deposits / vacuolation	0	0	0	0	0	0	0	0	0
		Intercellular deposits	MMC	0	0	0	0	0	0	0	0	0
		Nuclear alterations		1	1	1	1	2	2	2	2	4
		Atrophy		0	0	0	0	0	0	0	0	0
		Necrosis		0	0	0	0	0	0	1	1	1
	Glomerulus	Structural alterations		1	2	2	2	2	4	2	2	4
		Plasma alterations	Granular	1	1	1	1	2	2	1	2	2

			degeneration / intra cellular deposits / vacuolation									
		Intercellular deposits	MMC	0	0	0	0	0	0	0	0	0
		Nuclear alterations		1	1	1	1	1	1	1	2	2
		Atrophy		1	1	1	1	1	1	1	1	1
		Necrosis		1	1	1	1	2	2	2	2	4
			RP INDEX			**14**			**25**			**32**
PC	Tubule	Hypertrophy	e.g. albuminous degeneration (reversible) (cloudy swelling)	0	0	0	0	0	0	1	1	1
		Hyperplasia		1	1	1	1	1	1	1	2	2
	Interstitial tissue	Hypertrophy		0	0	0	0	0	0	0	0	0
		Hyperplasia	e.g. cirrhosis	0	0	0	0	0	0	0	0	0
	Glomerulus	Hypertrophy		1	1	1	1	1	1	1	1	1
		Hyperplasia		0	0	0	0	0	0	1	1	1
		Thickening of BC membrane		0	0	0	0	0	0	0	0	0
			RP INDEX			**2**			**2**			**5**
I		Exudate		0	0	0	0	0	0	0	0	0
		Activation of RES		0	0	0	0	0	0	0	0	0
		Infiltration	e.g. leucocytes (MNL) - lymphocytes	0	0	0	0	0	0	0	0	0
			e.g. granulocytes	0	0	0	0	0	0	0	0	0
			RP INDEX			**0**			**0**			**0**

Discussion

In the present study, an attempt is made to semi quantify the histological alterations in gills, liver and kidney on PE exposure. Sub lethal exposure of a variety of herbicides has been reported earlier and they have observed mild to severe damage in various tissues (Muthukumarave *et al.,* 2013, Tilak *et al.,* 2005; Chattopadhay *et al.*,2006; Farombi *et al.*,2008; Omitoyin *et al.*,2006; Srivastave *et al.,* 2008; Ladipo *et al.,* 2011; Khoshnood and Khoshnood, 2014; Ahmadivand *et al.,* 2014; Samanta *et al.,* 2016). The histology of gills of *O. mossambicus* appeared relatively normal in control as compared to the PE treated fish. The major changes in gills were regressive changes (epithelial lifting, thickening of the epithelial of primary lamellae and fusion of secondary lamellar), furthermore hyperplasia and hypertrophy and an increase in mucous secretions was also observed in the fishes exposed to higher dose of PE. Fish gills are the dominant site for gas exchange, ion regulation acid-base and nitrogenous waste excretion as well as toxicant uptake/detoxification, metabolism of circulating hormones (Chu *et al.*,2001; Rai and Mishra, 2014) Gills of fish constitutes upto 90 % of the total body surface area and are in intimate contact with the ambient water (Kumar *et al.,* 2015 and Lefevre *et al.,* 2017; Witzmann and Brainerd, 2017) , thus, any exposure of the contaminant from the water in turn makes the tissue vulnerable to the external environment and water born toxicant. Hence, when fish is exposed to environmental pollutants the vital functions are deleteriously affected and the functional impairment of the gill significantly damages the health of fish. PE exposure has probably resulted into the loss of adhesion between the epithelial branchial cells and the underlying pillar cells, accompanied by collapse of the structural integrity of the secondary lamellae and subsequent failure of the respiratory functions of the gills.

Fish gills are spectacularly equipped with a defence mechanism working against the environmental irritants which essentially is the mucus cell. The mucus cells react instantaneously to the pollutants and secrete copious mucus to form a thick protective layer over the entire exposed surface, which remain stuck to the mucus. The mucus layer creates a microenvironment that act as an ion trap, concentrating trace elements in the water. The histomorphological response of the gills of fish exposed to a toxicant is often manifested by a prominent increase in the density of its mucus cells (Kumar *et al.,* 2015). The large amount of mucous secretion in the present study has thus acted as a defence mechanism against PE (Parikh *et al.,* 2014). Furthermore, due to PE intoxication the gill epithelium was seen to be completely separated from the basement membrane and there was a swelling of the secondary lamellae and dilation of the vessels. The disorganized fusion in secondary gill epithelium was prominently observed after exposure of the PE. Histopathological change in the gill of *Nile Tilapia* was reported by Jiraungkoorskul *et al.,* 2003 and by Samanata *et al.,* 2016 in *Heteropneustes fossilis* after exposure of the fish to glyphosate herbicide. According to their investigation epithelial proliferation, congestion of blood vessel and hyperplasia of mucus cells was reported in the gills. The structural alterations in the gill morphology had been categorized by Dutta *et al.,* (1996) into two groups: (1) the insecticides causing necrosis and rupture of the branchial epithelium. These changes are dose dependent and often reported under lethal conditions. Our results on PE exposure are in agreement with the observation of Dutta *et al.,* (1996) that the alteration in the gill morphology was in a dose dependant manner. The alterations in the branchial cells and their rupture usually develops either by autolysis or by rapid lysis caused by the direct action of toxicants on the cells' constituents and (2) branchial defence response achieved by mucus

hypersecretion, epithelial lifting, swelling, hyperplasia and lamellar fusion(Kumar *et al.*,2016) . The increased mucus secretion also may be an adaptive strategy of the fish for the elimination of PE (Pandey *et al.*, 2011). The accumulation of the pesticide on gill imitated the elevation of mucus secretion and decreased ventilation which ultimately decreased the O_2 uptake through gills. The similar observation was reported by Prashanth *et al.*, 2011, Wannee *et al.*, 2002 and Wilson *et al.*, (2008) who had observed the epithelial lifting and aneurysm in the Nile tilapia, *Oreochromis niloticus* under exposure to glyphosate for 96h. The enlargement of chloride secreting cells and their nuclei supports the above assumption.

The liver is the central metabolic organ and plays a key role in biochemical transformations of the xenobiotic substances, which inevitably reflects on its integrity by creating lesions and other histopathological alterations in the liver parenchyma. In the present study, a variety of histopathological changes were observed in the liver of *O. mossambicus*. The severity and frequency of organ lesions was found to be more pronounced in fish treated with high dose of PE because of alterations were dose and time dependent. The first noticeable change was necrosis of hepatocytes, which were identified as a darkly stained eosiniphilic cytoplasm and was having a pyknotic nucleus, secondly glycogen vacuoles were identified as very small rounded open spaces within the cytoplasm of hepatocytes, Vacuolation was identified as round, empty spaces within the cell cytoplasm and the contents of the cells were most probably dissolved, the severity of the alterations were mild, moderate and severe with LD, MD and HD. Furthermore, nuclear pleomorphism was identified as nuclei being of different sizes and shapes and nuclear pyknosis was identified as nuclei which had densely compacted chromatin, smaller, stained very darkly, further, an increase in the number of melanomacrophage centres (MMCs) were identified as

groups of deposits with yellow-brown pigmentation within the liver tissue observed in HD of 14th day exposure period. Macrophages are related to the breakdown of erythrocytes, antigen presentation, iron storage and detoxification (Marcon et al.,2015). Taxins can cause increase of macrophage in the liver (Pascoli *et al.,* 2011 and Boran *et al.,* 2012), thus, macrophage provides evidence of absorption of exogenous substances, suggesting that the presence of these cells is indicative of toxicity at the sub acute exposure of PE after 14 days.

Several studies have reported accumulation of lipids in hepatocytes associated with toxic exposure (Glover *et al.,* 2007 and Sarma *et al.,* 2012); which changes the appearance of the liver tissue and a large spherical vessel in hepatocytes are seen and is characteristic of lipid deposition. The similar appearance of large spherical in *O.mossambicus* suggests that PE may have affected the lipid deposition. The presence of pancreatic cells distributed in the liver of *O.mossambicus* suggests that these cells have a role in detoxification. Altered structural integrity observed in endothelial and hepatopancreatic cells of *O.mossambicus* indicates that these cells are morphologically affected by PE.

The organ most associated with the detoxification and accumulation process is liver and due to its function, position and blood supply, it is also one of the organs most affected by contaminants in the water (Camargo and Martinez, 2007) it also plays a prominent role in fish physiology, both in anabolism (protein, lipid, carbohydrate) and catabolism (glycogenolysis, detoxification) and it acts as storage centre for many substances, mainly glycogen. The liver of the fish exposed to low, medium as well as high dose showed vacuolar degeneration, hypertrophy in the hepatocytes with nuclear pyknosis and karyopyknosis Ramah, (2011) due to apoptosis and fragmentation of the nucleus. These changes may be attributed to direct toxic effects of PE on hepatocyte as found

in other pesticide toxicity (Mohamed , 2009; Van Dyk *et al.*,2012; Desai *et al.*,,2014 and 2016; Robson *et al.*,2017 and Gerber *et al.*,,2017) because of it is the site of detoxification of all type of toxins and chemicals. The liver parenchyma showed sign of degeneration (cytoplasmic and nuclear), vacuolation of the hepatocytes (Pacheco and Santos, 2002), probably due to metabolic damage related to exposure of PE. Steatosis of the liver was visible as vacuoles resembling fatty degeneration and was observed at only at HD on 14 day. On closer inspection, the hepatic tissue surrounding the central vein was atrophied and the presence of nuclear pleomorphism was evident, further the observed abnormal abundance of RBCs in the sinusoids and blood vessels is indicative of sinusoidal congestion. Large groups of melanomacrophage centers (MMCs) were also observed, however it has been suggested in literature that these structures are a normal characteristic in fish tissue (Leknes, 2004). Thus, from the present investigation it can be concluded that PE has probably induced interactions between leukocytes and the endothelium, causing an increase in leukocyte movement from blood vessels into tissue as an inflammatory response (Dezfuli *et al.,* 2015). This may explain why fish exposed to PE also resulted in a significantly lower WBC values at high dose of PE on 14the day and thus the PE exposure may have elicited an inflammatory response causing leukocytes to leave the blood vessels and enter tissue, resulting in a lower WBC count (Robson *et al.*, 2017).

The kidney has a varied function among fish species and filters large quantities of blood and produce urine, which is the major route of excretion for some xenobiotic (Blazer, 2000, Thophon *et al.,* 2003). The kidney of fish receives the vast majority of postbranchial blood and therefore a renal lesion is expected to be a good indicator of environmental pollution. In freshwater teleost,

the main function of the kidney is to excrete the large amounts of water which enter the fish body through the gills (Cengiz, 2006; Tilak *et al.,* 2007).

The functional unit of the kidney is nephron. It is made up of following parts: The renal corpuscle (consisting of a glomerulus and Bowman's capsule) and the renal tubules consist of a single layer of epithelial cells. The renal corpuscles are surrounded by cross sections of proximal convoluted tubules. The lumen of the distal tubule is more rounded and the apical surface of the cells was sharper. The cells forming the collecting tubules are cuboidal and smaller than those of proximal tubule.

Renal tissues of the *O.mossambicus* exhibited sub-lethal toxicity of PE which was evidenced by marked histopathological alterations. The alteration in the histological features of kidney was dose and time dependent, however, the severity and frequency of organ lesions was found to be more pronounced in fish treated with HD of PE. In *O. mossambicus*, the waste products and non-detoxified herbicide molecules gets eliminated through kidney hence, it is susceptible to chemical compounds (Genten *et al.,* 2009 and Deivasigamani, 2015), sub-lethal exposure of PE has resulted into degenerative changes in renal tubule and glomerulus. As far as the corpuscles are concerned, there was a distinct dilation of the glomerulus capillaries as well as glomerular enlargement. In the tubules, nuclear hypertrophy along with cellular hypertrophy was seen; there were cellular vacuolation, cloudy swelling and dilation of the tubular lumen. Other then this alterations the decreased Bowman's space resulted into infiltration of the blood in the Bowman's space. The severity of lesions were highly pronounced with HD on the 14th day, which include severe necrosis, cloudy swelling in renal tubules, cellular hypertrophy and granular cytoplasm. The epithelial cells of the distal convoluted tubule decreased in size. Renal interstitial tissue

showed formation of vacuoles and cellular contours were not clearly distinguished, similar results were obtained by Kathiresan and Ramah (2002); Bharat Bhusan Patnaik *et al.*,(2011) and Reza Sayrafi *et al.*,(2011); Gabi Dumitrescu *et al.*,(2010) on the kidney by exposure of herbicides.

Furthermore, our result are in agreement with Ramírez-Duarte *et al.,* (2008) and S. Deivasigamani (2015) where they have monitored toxicity of the glyphosate in *Piaractus brachypomus* and *Cyprinus carpio* and have reported the histopathological lesions in kidney with vacuolar degeneration of tubular epithelium, desquamation of epithelium and necrosis of tubular epithelium. Ahmadivand *et al.,* (2014) have also reported histological alterations in the kidney of *Oncorhynchus mykiss* exposed to butachlor. Moreover, hyperplasia and appearance of melanomacrophagic centers (MMC) is reported by Salazar-Lugo *et al.,* (2011) in *Colossomama cropomum* exposed to paraquat at different temperatures.

The abnormalities observed in present study such as necrosis of renal tubules, desquamated epithelial cells, atrophy of glomeruli and hemorrhages suggested that PE must have entered the kidney which resulted into a disruptions of their normal functioning. The necrosis of renal tubules can in turn affect the metabolic activities of the kidney promoting metabolic abnormalities in *O.mossambicus.* All the alterations grouped together points to the fact that the kidney exhibited regressive changes. Regressive changes are related to a reduction of the organ function, which may lead to inhibition of the physiological function of the entire organ, (Van Dyk, 2006, Marchand *et al.,* 2012, Nibamureke *et al.,* 2016). In view of the studies cited above, it is apparent that in the present investigation, PE at sub lethal concentrations caused considerable histological damages to the organs studied.

Histological alterations were confirmed by the semiquantitative analysis, which includes circulatory disturbances, regressive changes and progressive alterations. On the basis of the reaction pattern, the organ index of all the tissues after 7 days of PE exposure exhibited a slight histological alteration which represents class I. However, at low dose; Gills, liver and Kidney exhibited moderate histological alterations which represent class II. At medium dose, all the tissues exhibited severe histological alterations which represent class III, and at High dose, Gills, Liver and Kidney exhibited pronounced histological alterations which represent class IV. Overall health status of *O.mossambicus* revealed that PE.

In conclusion, the present study indicates that sub acute exposure to PE induces histopathological and ultrastructural changes in gills, liver, and kidneys. The cytopathological lesions in all three tissues recorded under two different time period and three different concentration exposure demonstrate the cumulative physiological and biochemical effects of PE. Overall, it is concluded that more or less similar pathological changes are induced in the kidney *O.mossambicus* by different concentration as well as different time period suggesting that of all the tissues kidney was more vulnerable to PE.